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## Isothermal titration calorimetry of RNA

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## ABSTRACT

Isothermal titration calorimetry (ITC) is a fast and robust method to study the physical basis of molecular interactions. A single well-designed experiment can provide complete thermodynamic characterization of a binding reaction, including  $K_a$ ,  $\Delta G$ ,  $\Delta H$ ,  $\Delta S$  and reaction stoichiometry ( $n$ ). Repeating the experiment at different temperatures allows determination of the heat capacity change ( $\Delta C_p$ ) of the interaction. Modern calorimeters are sensitive enough to probe even weak biological interactions making ITC a very popular method among biochemists. Although ITC has been applied to protein studies for many years, it is becoming widely applicable in RNA biochemistry as well, especially in studies which involve RNA folding and RNA interactions with small molecules, proteins and with other RNAs. This review focuses on best practices for planning, designing and executing effective ITC experiments when one or more of the reactants is an RNA.

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## 1. Introduction

Isothermal titration calorimetry (ITC) is a powerful and versatile method to study the physical basis of molecular interactions. In an ITC experiment, one physically measures the heat generated or absorbed during a binding reaction ( $\Delta H_{\text{obs}}$ ). The typical experiment involves addition of one binding partner (titrant) into the other binding partner (titrate) over time using one or more individual injections. Depending on the type of instrument being used, heat is measured either as a change in temperature or as the change in power required to maintain temperature between the sample and a reference cell. This energy is then converted into a binding enthalpy based on knowledge of the cell volume and the concentration of the reactants. The observed enthalpy measured in an experiment includes not only the heat of binding between the molecules, but also any additional heat sources associated with the reaction including solvent effects, molecular reorganization and conformational changes, heats of dilution and purely mechanical artifacts such as heat resulting from sample stirring. Thus, careful preparation of solutions and measurement of appropriate background heats are required to obtain thermodynamic parameters that accurately reflect the event(s) of interest.

The experiment can be performed as either a continuous or sequential titration. The heat produced during each injection is proportional to the amount of complex formed. Thus, one expects maximal enthalpies at the earliest points in a titration with a decrease in intensity as free titrate is consumed. The change in

heat over the course of the titration will give rise to the binding stoichiometry and affinity constant. Since  $K_a$  is related to the Gibbs free energy ( $\Delta G$ ) of binding and  $\Delta H$  was measured directly, reaction entropies ( $\Delta S$ ) can be readily calculated from a single measurement without relying on van't Hoff approximations. By repeating the experiment over multiple temperatures, heat capacity changes ( $\Delta C_p$ ) for the reaction can also be easily measured.

The ability to obtain complete thermodynamic characterization of a reaction quickly and accurately from a single ITC measurement has made the technique very popular. Over the past two decades, calorimeters have improved immensely in terms of sensitivity and ease of use [1], which makes modern ITC versatile enough to explore interactions in biological systems. The sensitivity of the modern instruments has tremendously expanded the range of affinities over which measurements can be made, spanning from the low millimolar range for weak binders to sub-nanomolar interactions on the tight end. In certain cases one can extend the range of accessible affinities even lower if there are families of compounds that can be used when competition methods are employed (discussed in more detail below) [2].

In general, three types of ITC experiments have been performed on RNAs based on the types of binding interactions being analyzed. These include RNAs binding to: (a) small molecules or ions; (b) proteins or (c) other RNAs. For the most part we will treat these equivalently as the experimental design considerations are pretty similar. Several recent reviews describe modern ITC instrumentation and its application in biochemistry and biophysics [3–8]. This review will focus on more practical aspects of performing effective ITC experiments on RNAs with only a limited discussion of the theory and instrumentation.

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## 2. Examples of RNA ITC experiments

Calorimetric analysis of RNAs is a growing field due to ready availability of the instrumentation in core facilities and reduced sample requirements of modern calorimeters. Of the three types of RNA interactions found in the ITC literature, small molecule interactions are among the most prevalent studies due to their relative simplicity from a conceptual perspective. Analysis of the specificity of riboswitches lends itself nicely to characterization by calorimetric methods, illustrated by the work from the Batey Lab among others [9–11]. Aminoglycoside binding to a variety of RNAs has also been prominent in the ITC literature [12–15] and unraveled thermodynamic relationships that could be important in characterizing and optimizing the drug-target interactions for this class of antibiotics.

A variety of RNA–protein interactions have been studied recently using ITC. The Puglisi's group used ITC to probe how structural features of the natural RNAs such as bulges and loops affect RNA binding to the double-stranded RNA binding domains (dsRBDs) of PKR, a double-stranded RNA-dependent protein kinase [16]. This particular study revealed using HIV TAR RNA that bulges and loops are essential for high affinity binding to the dsRBD domains. In another study, ITC was used to look at the interaction between the *Escherichia coli* protein Hfq and one of its RNA binding partners, DsrA, a non-coding RNA induced in response to cold shock [17,18]. ITC was particularly important in this study for assaying the stoichiometry of the binding. ITC has also been used to study the thermodynamics of the assembly of individual subunits in the 30S ribosomal subunit in bacteria [19]. In this work ITC was used to show that S8 and S11 are thermodynamically independent of the other proteins, while S15 had a cooperative effect on the S6/S8 heterodimer. ITC was also able to probe the sum of individual protein binding steps measured in isolation, proving the aggregate value was comparable to the binding that was performed using all components in a single injection, supporting the deconstructive approach in studying ribosomal assembly.

Understanding of fundamental RNA folding thermodynamics has also benefited from the application of ITC. Nearest-neighbor models which derived predominantly from UV-melting studies have been used extensively with great accuracy in RNA secondary structure prediction [20,21]. The main advantage that ITC provides is a direct measurement under a specific temperature condition that reveals non-two state behaviors lost into the baseline corrections of UV-melting traces. One such parameter is the heat capacity change ( $\Delta C_p$ ) that significantly affects enthalpic and entropic contributions of RNA and DNA folding but is often neglected due to its more modest effect on  $\Delta G$ , a result of entropy–enthalpy compensation [22–24]. Furthermore, RNA tertiary rearrangements that exhibit little UV-hyperchromicity like kissing hairpin interactions or junction folding can be observed by ITC [25,26]. These studies are allowing the thermodynamic dissection of more complicated RNAs and beginning the process of parameterizing non-duplex contacts in RNA structures. The ability of ITC to effectively analyze non-two-state systems and to probe interactions that are unobservable by other methodologies makes it a powerful tool for the on-going characterization of RNA folding.

## 3. Instrument description and methodology of ITCs

The most widely available ITCs used for microcalorimetry of biological samples are based on the power-compensation methodology. Both the MicroCal VP-ITC and the Nano-ITC formerly produced by Calorimetry Sciences Corp. (purchased in 2007 by TA Instruments, a subsidiary from the Waters Corporation) use this technology. The advantages of this style of instrument include its

fast response time, high sensitivity and modest sample requirements. A schematic diagram of a typical ITC is shown in Fig. 1. Two equivalent lollipop shaped cells are suspended in an adiabatic jacket. The temperature of each cell is monitored and maintained at a constant temperature through an electronic feedback loop that controls thermoelectric heaters located adjacent to each cell. During the experiment the titrant, which is in the injection syringe, is added to the sample cell. If the reaction is exothermic, heat evolves and  $\Delta T_1$  increases. The feedback loop responds by reducing the power to the resistive heater around the sample cell to restore  $\Delta T_1$  to zero. Thus, in a power versus time curve, the injection results in a negative deflection from the baseline, the integrated area of which corresponds to the total enthalpy released as a function of the injection (Fig. 2, top panel). An endothermic reaction would result in a corresponding positive peak. At saturation when no reaction is taking place, the baseline reflects a constant power consumption proportional to the power needed to maintain the temperature differential between the cell and the adiabatic jacket ( $\Delta T_2$ ). Small peaks are often still observed due to effects such as buffer mismatch between the titrant and titrate or non-specific binding. After integration and background correction, one obtains a thermogram (Fig. 2, bottom panel) that can be fit to a specified binding model.

The example ITC data shown in Fig. 2 is from an experiment in which the energetics of RNA duplex formation was measured. The titrate was a 7-nt RNA (5'-CGUCC-3') and the titrant was its complement. Both samples were prepared by dilution of a concentrated RNA stock solution in water into buffer (50 mM Hepes, pH 7.5, 1 M NaCl) and degassed prior to use. In this case the dilution method was required rather than dialysis due to the small size of these RNAs. Small amounts of residual salt in the RNA stock solutions from ethanol precipitation would be only a minor issue here due to the high NaCl concentration of the binding buffer. Data were collected during forty 7  $\mu$ L injections of 75  $\mu$ M titrant into 5  $\mu$ M titrate at 25 °C with stirring at 310 rpm and 240 s injection spacing. Equivalent data were collected when the titrant RNA was used as the titrate. The raw data were integrated and normalized using ORIGIN (MicroCal Inc., ver. 7.0) resulting in a plot of  $\Delta H$  per mole of injectant versus molar ratio. This plot was fit to a simple binding model.

Measurement of background heats is a critical aspect of the ITC experiment. There are two standard ways in which to perform this correction and one must decide in advance which method is to be used as it impacts the experimental design. The most common

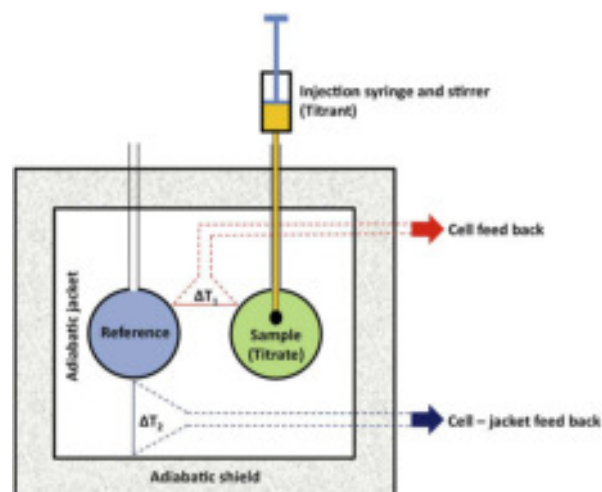
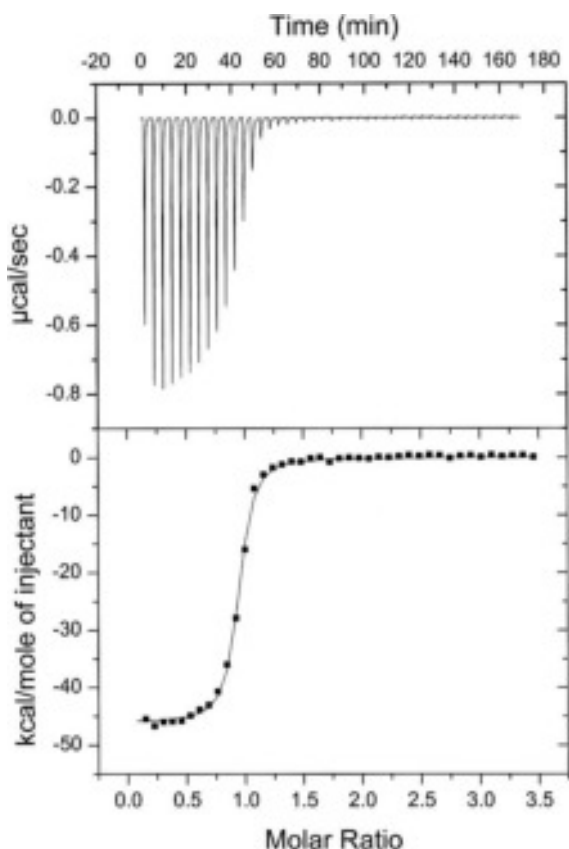


Fig. 1. A schematic diagram of a typical energy compensation isothermal titration calorimeter.



**Fig. 2.** Example ITC data from an experiment measuring the energetics of RNA for duplex formation. A 7-nucleotide single-stranded RNA was titrated into its complement in 50 mM Hepes pH 7.5, 1 M NaCl at 25 °C. (Top panel) Power versus time curve. (Bottom panel) Thermogram of the integrated peak intensities plotted against the molar ratio of the two strands. Fitting of this titration yielded a  $K_d = 4.3 \times 10^7 \text{ M}^{-1}$ ,  $\Delta H = -46.1 \pm 0.2 \text{ kcal/mol}$  and  $n = 0.91 \pm 0.01$ . Data reproduced with permission from Ref. [24] (Copyright, American Chemical Society).

method is to use a series of blank injections. This involves repeating the exact same injection protocol but with buffer in the cell rather than titrate. Thus, one sees the effects of diluting the titrant and any heats involved in that process. The disadvantage of this method is

that it is time consuming since the entire titration protocol is typically repeated. The second method is to extend the titration to collect extra data at the end of the experiment under saturation conditions. This involves having additional titrant available to collect 5–10 extra injections after all of the titrate has been bound. One then prepares a linear extrapolation of the heats from these final injections to apply as a correction over the course of the entire experiments. This latter method of background correction was used in the ITC experiment shown in Fig. 2 which is why so many additional data points were collected after saturation was achieved. Both methods provide equivalent results in our hands so long as there is little non-specific binding of the titrant to the titrate.

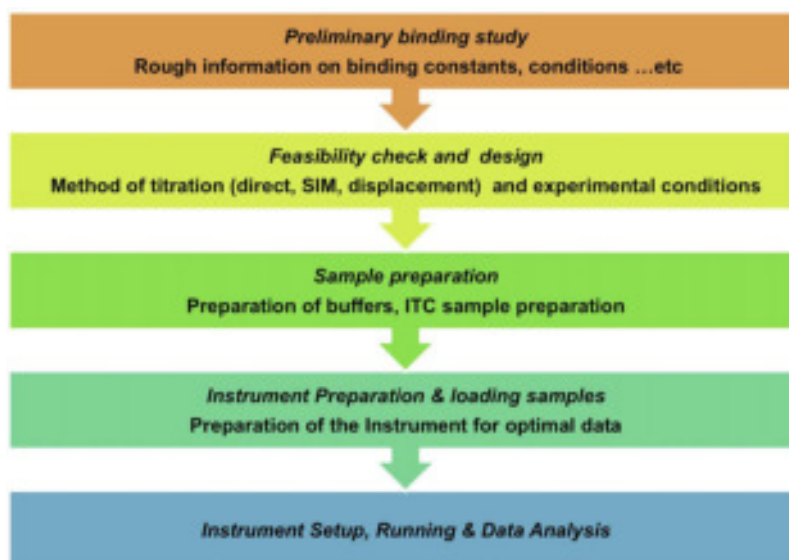
#### 4. Planning an ITC experiment

The basic protocol for ITC experiments is similar for the three classes of ligands described above. Thus, unless explicitly stated, the protocols should be regarded as common to all three categories of interactions. A flow chart illustrating the basic design of an ITC experiment is depicted in Fig. 3 and will serve as the focal point of this discussion. Where there are differences between the classes of interactions, they typically arise due to issues such as differential solubility of proteins or small molecules and the solution conditions under which such species are stable. Also, issues relating to non-specific binding and stoichiometry must also be recognized and addressed during the experimental design. This section will describe the planning that needs to be done to maximize the likelihood of performing the experiment successfully.

##### 4.1. Preliminary binding studies

Even with the development of microcalorimeters, the samples required to perform ITC analysis are large relative to many other methodologies (i.e. UV-melting, gel shift assays, surface plasmon resonance, etc.). With that in mind, it is prudent to perform preliminary binding studies on a new system before filling the ITC cell with an expensive RNA.

The first consideration has to do with whether or not the RNA is well-behaved. Does the material exhibit alternative conformations on native gels or are there problems with monomer–dimer equilibria for instance? These types of equilibria will make it next to impossible to analyze and interpret the ITC data and should be re-



**Fig. 3.** Overview of an ITC experiment.

solved prior to initiating ITC studies. While methods of resolving these issues are relatively standard, one must remember that the concentrations used in the calorimeter are much higher than for many other types of experiments. Monomer–dimer equilibria are dependent on the concentration and one should perform small scale refolding studies at the target concentration you will use in the ITC. Hairpins that are well-behaved at low concentrations used with  $^{32}\text{P}$ -labeled material may not be equally well-behaved when annealed at micromolar concentrations. Furthermore, one should evaluate sample homogeneity under the highest and lowest salt or  $\text{MgCl}_2$  conditions one plans on using in the calorimeter to ensure that the calorimetry studies do not simply reflect changes in alternative folding of the reactants.

For RNA–RNA and RNA–protein interactions, gel shift assays will often be the best choice for preliminary studies. They require minute samples and allow evaluation of binding affinities. Furthermore, because gel shift assays provide spatial separation of the products, this preliminary experiment will also alert the user to potential problems associated with product heterogeneity. Is there a single product forming under a given condition or are there multiple bands present representing the formation of higher order complexes that might complicate ITC data analysis?

For small molecule interactions, preliminary binding studies are often not as simple as they are for macromolecular interactions. Rarely do the small molecule interactions provide large mobility changes upon binding making gel shift assays less useful. Several biophysical approaches can still be used however, depending on the system. A generic methodology that is reasonably effective is to look at  $\Delta T_M$  in UV-melting analysis upon binding [27]. Since the method requires no extrinsic labels or significant structural changes upon binding, it is quite versatile. Other approaches such as circular dichroism [27] or fluorescence anisotropy [28] can be used, but their utility is commonly dependent on the exact nature of the RNA and the binding partner being studied.

Since the intent of these preliminary studies is preparation for ITC analysis, we need to make a quick statement regarding buffers at this point. When selecting experimental buffer conditions for the ITC, most common salts and buffer additives are acceptable. The one notable exception is DTT, however, which one periodically finds as a reductant in protein storage buffers. DTT concentrations as low as 1 mM can cause severe baseline artifacts due to background oxidation during the titration. If possible, we recommend removing all reducing agents by dialysis prior to performing ITC analysis. If they must be present for stabilization of a reactant, one should consider purging the system with argon or nitrogen prior to degassing to avoid problems with autoxidation.

#### 4.2. Considerations that effect sample concentration

Once preliminary binding studies are complete and the system shows good homogeneous behavior, the next step is to determine exact conditions that will be used in the ITC. For most people embarking on an ITC study for the first time, they are most interested in knowing, how much material they need to prepare and at what concentration? While ITC instruments are much more sensitive than they used to be, these studies still require large samples. The knee jerk answer to this question for our instrument (a VP-ITC from MicroCal) is that one typically needs about 1.8–2 mL of titrate solution in the low micromolar range (typically 1–2  $\mu\text{M}$ ) while the titrant should be  $\sim 400\ \mu\text{L}$  of a solution 15–20 times the concentration of titrate. Other instruments have different cell volumes, so inquire regarding the specific ITC you plan on using prior to preparing your samples. There are more precise ways to determine sample concentration. To make that determination, however, one needs to know a little more about how a thermogram is analyzed and a parameter called the  $c$ -value [1].

A typical ITC thermogram was shown in the top panel of Fig. 2. As explained above each deflection from the base line is a result of the change in the differential power that is required to maintain the sample and the reference cell at the same temperature. As the amount of heat absorbed and emitted are equal to the change in the power, the heats of each injection ( $q_i$ ) can be obtained by integrating the respective peak and mapping it to the fractional saturation of the binding reaction ( $F$ ) based on the stoichiometry ( $n$ ), the total concentration of the titrate ( $M_T$ ), the binding enthalpy ( $\Delta H$ ) and the cell volume ( $V$ ), when the concentration of the titrant ( $B_T$ ) is known [1,6,29,30] (Eqs. (1)–(3)).

$$q_i = nFM_T\Delta HV \quad (1)$$

$$F^2 - F \left\{ 1 + \frac{B_T}{nM_T} + \frac{1}{nK_B M_T} \right\} + \left( \frac{B_T}{nM_T} \right) = 0 \quad (2)$$

$$q_i = nM_T\Delta H \left( \frac{V}{2} \right) \left\{ X - \sqrt{\left( X^2 - \frac{4B_T}{nM_T} \right)} \right\} \quad (3)$$

where

$$X = 1 + \frac{B_T}{nM_T} + \frac{1}{nK_B M_T}$$

From Eq. (1) one can solve for  $F$  and substitute into Eq. (2) to obtain a quadratic relationship, which then can be solved for  $q_i$  given Eq. (3). Since  $B_T$  is known, one can now fit the solution into a non-linear least squares analysis to solve for thermodynamic parameters such as  $\Delta H$ ,  $K_B$ ,  $n$  where  $K_B$  is the binding constant of the reaction. Once  $K_B$  is obtained  $\Delta G$  can be calculated using Eq. (4) and  $\Delta S$  from Eq. (5).

$$\Delta G = -RT \ln(K_B) \quad (4)$$

$$\Delta G = \Delta H - T\Delta S \quad (5)$$

In the solution for  $X$  in Eq. (3), one sees the product  $n \times K_B \times M_T$  which was named the  $c$ -value and is a measure of the sigmoidicity of the curve. Wiseman et al. showed that to obtain useful information from the titration,  $1 < c < 1000$  and ideally  $c$  would be between 10 and 100. Therefore, for weak interactions, while one might be tempted to increase the titrant concentration, this may lead to the loss of information as the whole reaction will occur within a single titration point and  $K_d$  would be poorly defined. On the other hand for stronger binding systems lower concentrations are preferred. Here the limitation will be the amount of heat given off by the reaction. When this signal becomes too small, the transition is hard to resolve above the background and the effective error in the resulting thermodynamic parameters increases [31]. So, if you have a ballpark affinity and a notion regarding the stoichiometry, one can simply calculate  $M_T$  for  $c = 10$  and 100. Tellinghuisen showed based on a theoretical analysis that one should optimally set the terminal ratio of  $B_T/M_T$  based on Eq. (6).

$$R_m = \left( \frac{B_T}{M_T} \right) = \frac{6.4}{c^{0.2}} + \frac{13}{c} \quad (6)$$

In practice, for a typical titration involving addition of  $\sim 280\ \mu\text{L}$  titrant into 1.4 mL titrate with a 1:1 stoichiometry, this yields a 30-fold concentration ratio to achieve  $c = 10$  and 15-fold ratio for  $c = 100$ .

#### 4.3. Who should be titrated into whom?

When designing an experiment in ITC, one of the first questions is often who should be titrated into whom? This is one of the cases where one has to distinguish between the three common types of RNA experiments. For RNA–RNA interactions, solubility is rarely an

issue and to a great extent it does not typically matter which species is in the cell and which is in the syringe. In fact, one commonly repeats the experiment by flipping the two to ensure that there are no differences which would reflect problems with alternative conformations during annealing at high concentrations. The situation is not so clear-cut for protein or small molecule interactions. Here, the primary concern is often one of solubility. The experiment is often easiest if the least soluble material is used as the titrate as this prevents precipitation or aggregation that might skew the data.

#### 4.4. Sample preparation

As with all thermodynamic measurements on RNA, sample quality is of utmost importance. It makes little difference whether the material derives from *in vitro* transcription or chemical synthesis. Typically RNAs will be either gel purified or HPLC purified prior to use. The critical issue is that the material is as homogeneous as possible and folds into a single conformation. The preliminary studies described above will have shown that the annealing conditions are appropriate for the desired experiment.

Buffer match between the titrate and titrant solutions is a critical aspect of sample preparation. How one achieves this will be dependent upon the size of the RNAs in question and the ligands with which they will be interacting. If both species are sufficiently large, the solution is simply to dialyze them in a common reservoir. This will ensure that the two samples are rigorously identical. Perform the dialysis at high RNA concentration so that some dilution during dialysis is not a problem and then adjust the final concentration of the RNAs by diluting them with dialysate. If one or both of the RNAs is too short to be retained during dialysis, one can also prepare them by precipitation, followed by resolubilization in the appropriate buffer. The potential problem here is that one must be careful to wash the RNA pellet very effectively as any residual salt from the precipitation will induce a buffer mismatch. While background correction will be used to account for small discrepancies, it is easy to incorporate significant errors as a result of this method. For small molecule ligands, it is common to dissolve them in the dialysate from the RNA buffer exchange. Once the samples are prepared they need to be degassed before being loaded into the ITC instruments. Typically we degas at a few torr partial pressure for 5 min in the thermovac instrument supplied by MicroCal. Inadequate degassing will produce large spikes in the ITC baseline due to the formation of air bubbles during the experiment, which adversely affects data quality. One should be consistent with this degassing step as it has the potential to alter sample concentrations. If this is a concern, one can remeasure the concentration after loading the sample into the syringe/cell using the excess material leftover from filling the instrument.

It was mentioned above that DTT and other reductants should be avoided during ITC experiments. Another issue to consider carefully is sample pH. Remember that ITC measures total heat ( $\Delta H_{\text{obs}}$ ), which comprises the binding event, solvent dilutions as well as ionization/deionization enthalpies. If changes in protonation state accompany binding or folding, a buffer dependent contribution will be superimposed on the data where the magnitude is dictated by the protonation enthalpy of the buffer and the solution pH. This was illustrated nicely in a study of aminoglycoside binding to 16S rRNA where pH and buffer sensitivity was observed [14,32,33]. By repeating the ITC experiments under several buffer conditions the contribution from the buffer component can be factored out [6]. One should routinely use control experiments to assess potential buffer linkage, by repeating the experiment in a different buffer and/or at a different pH.

For experiments involving nucleic acids, metal ions are often needed for proper folding. The metal ion–RNA interactions and their rearrangements as a function of the binding reaction is an

integral component of these systems. For this reason, one should expect a certain degree of linkage between the ionic conditions used in an experiment and the binding thermodynamics. Whether or not this contribution is significant will be highly dependent on the specific interaction being studied.

#### 4.5. Instrument decontamination

Fig. 4 provides a flow chart of the steps leading to ITC data collection. Cleaning the ITC is an important part of this process. This step is of particular importance to RNA users and is a step that should not be underestimated or taken lightly, especially if the instrument is part of a shared facility and used by non-RNA laboratories. The critical issue here is one of nuclease contamination. As a consequence of early work on power-compensation microcalorimeters, it has become standard in the field to use the binding of CMP to RNaseA as a standardization/quality control protocol and for training new users of VP-ITC instruments. This is a particularly dismal choice for those of us whose samples are readily degraded by this protein. For anyone considering using a shared ITC instrument, I would immediately inquire with the facility manager regarding this practice and urge them to change to a more benign standard as decontamination is a serious challenge. In our laboratory, for instance, we typically use the interaction between Ni(II) and histidine as our test system [34].

A typical cleaning protocol for the ITC cell entails soaking the cell with commercial detergents like 10% TopJob or Mr. Clean at room temperature or at 65 °C followed by extensive flushing with deionized water. Stringent cleaning involves a harsher detergent (5–20% Contrad-70), also followed by flushing with water. Unfortunately, neither of these treatments adequately removes nuclease contamination. If RNase has ever been used in the system or if non-RNA users have potentially contaminated the cell and syringe, one *MUST* decontaminate the instrument prior to use. Plan well in advance as this process can take as much as a week to complete depending on the severity of the contamination. Typically, we soak the ITC cell and syringe in neat RNase Zap<sup>®</sup> (Ambion) overnight at room temperature and then rinse the cell with 1 L of water. After this treatment, the ITC cell can be tested for nuclease contamination by letting an RNA sample stand in the cell for several hours before removing it and assaying it for degradation by PAGE. This process can be repeated several times and interspersed with treatment of the cell with 0.1% SDS at 65 °C or 0.1% pepsin at room temperature. Do not proceed until your sample is stable against degradation overnight.

Separate from RNase contamination that leads to wholesale degradation of the sample, a dirty ITC cell will lead to poor data quality. The common signature of this problem is significant baseline drift during ITC runs. Although minor drifts in the baseline (upward or downward) can be expected and corrected during data analysis, severe drift, often with non-linearity, is difficult to fit. Another indication of contamination in the cell is the reference power at the start of a titration. During an experiment the raw ITC data is plotted as the differential power ( $\mu\text{Cal/s}$ ) versus time (s). The user sets the reference power at the beginning of a titration. If one sets the reference power to 30  $\mu\text{Cal/s}$  this setting means that 30  $\mu\text{Cal/s}$  is continuously supplied to the heater around the reference cell. If the ITC is behaving well and the cell is clean, the baseline in the raw differential power plot should be close to this value—most often 1–2  $\mu\text{Cal/s}$  lower than the set point. If the initial baseline is considerably different than the reference power one should clean the cell prior to filling it with a precious RNA sample.

Once the user is confident that the cell is clean and free of nuclease contamination, the samples can be loaded into the cell and syringe, respectively. This is a step that requires a certain amount of practice and dexterity to keep the sample bubble free.

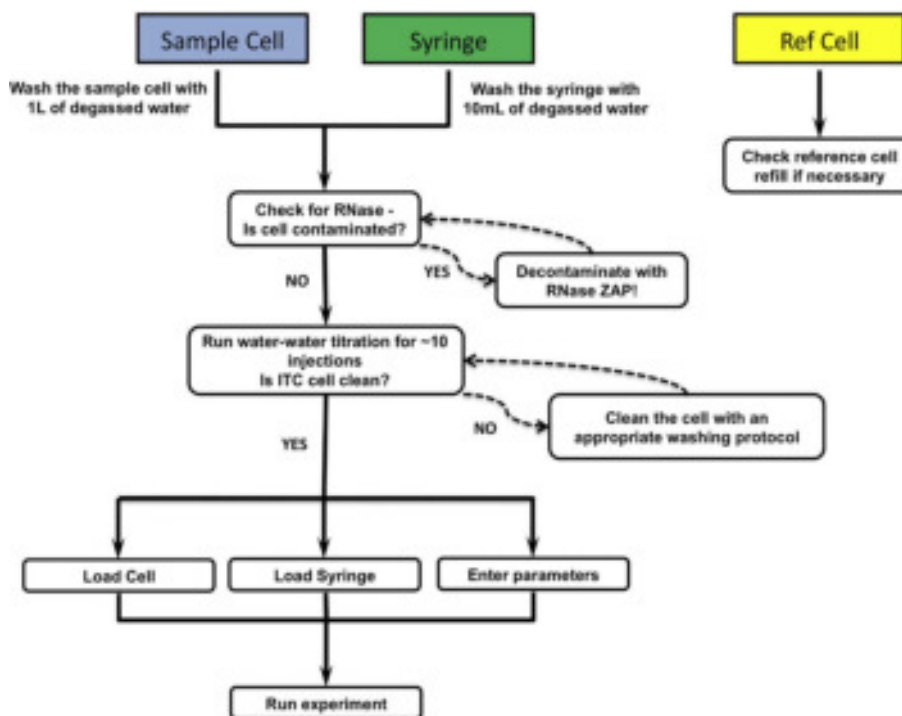


Fig. 4. Preparation of the ITC instrument for an experiment involving one or more RNA substrates.

Training new users with water or buffer is highly encouraged before moving on to more expensive RNA samples. Because buffer mismatch is of critical concern, it is important to rinse the cell and the syringe with the binding buffer prior to loading experimental samples. Both the cell and syringe should be thoroughly emptied to avoid diluting the experimental samples although a tiny amount of dilution is inevitable. Even though the titrant syringe only holds  $\sim 280 \mu\text{L}$ , plan on having  $\sim 400 \mu\text{L}$  of titrant solution to load the instrument easily and keep bubbles from forming inside the barrel.

#### 4.6. Instrument settings for data collection

There is a modestly large number of experimental parameters that need to be set by the user at the start of an ITC experiment. Joel Tellinghuisen has studied the systematic errors associated with ITC experiments and the manner in which these settings affect the data quality [31,35,36]. He has put forth some excellent guidelines regarding certain parameters that have particularly significant impact on the data. Three simple rules include: (a) minimize the number of total number of injections, (b) maximize the starting concentration of the titrate in the cell ( $M_T$ ) and (c) use Eq. (6) to set the titrant concentration to keep the  $c$ -value between 10 and 100. These are good guidelines to follow when starting out, but they require preliminary knowledge of the approximate association constant as discussed above. All of the adjustable parameters for a VP-ITC are listed in Table 1 together with some initial recommendations for the ITC novice. In addition, the table includes some notes regarding artifacts or issues that might arise if a given parameter is set inappropriately.

The injection volumes and the delay time between injections are two of the most asked about variables. It is very common if you look at ITC data that the first peak appears aberrantly small. Mizoue and Tellinghuisen have shown that this first injection anomaly results from a volumetric error due to the backlash in the motorized screw used to drive the syringe plunger [30]. Most people set the first injection to a very small volume (typically 1–

$2 \mu\text{L}$ ) and simply discard this point prior to data analysis to fix the issue. Alternatively, you can use the software to drive the syringe forward a couple of microliters prior to loading it into the cell for the start of the reaction. If you do this, be sure to wipe off the tip of the syringe to remove that material prior to inserting the needle into the ITC cell. The volume of subsequent injections needs to be set so that: (a) a significant amount of heat is being produced by each injection and (b) a reasonable number of points are situated in the transition region of the titration curve. This will be highly dependent on the system being studied. If cell concentrations are low or the reaction enthalpy is small, larger injections will be required to obtain a suitable signal. Most people use constant volumes for all injections after the first one. This is more of an aesthetic issue than a scientific one. The user also needs to set the spacing time between injections. The critical issue here is that the system must return to a good baseline after every injection. Failure to achieve this will adversely affect the data quality due to poor integration of the peak area. A good starting point is usually around 300 s. This time can be shortened if the injection peak returns to baseline rapidly and it can be lengthened if a flat baseline between injections is not achieved. Setting the injection spacing arbitrarily high can adversely effect your data due to baseline drift and also makes your experiment unnecessarily long which is important if you are being charged by the hour for use of a facility instrument. For most of the studies a stirring speed in the range of about 260–310 rpm works well and provides a stable baseline. For binding systems that have  $c$ -values greater than 1000 a higher stirring speed needs to be maintained. (Note: under these conditions, the reaction enthalpy can be measured but the value for the association constant will not be accurate.) If the sample is viscous stirring speeds will have to be adjusted so that a suitable baseline is obtained.

The last experimental parameter that needs to be set is the reference power. This value is essentially the amount of power that is used to heat the reference cell at the resting state and dictates the maximum heat that can be accommodated during a single injection. Setting the value too high can lead to a loss in instrument sen-

**Table 1**  
Typical experimental parameters for an ITC experiment<sup>a</sup>

Experimental parameter	Description	Setting	Comments
Cell temperature	The desired reaction temperature	2–80 °C <sup>b</sup>	Cooling the sample below the experimental temperature reduces ITC equilibration time, when <i>No check temp</i> option is off
No of injections	Total number of injection made throughout the experiment	25–35 <sup>c</sup>	The number of injections depend on the concentration the user sets, having considerable amounts of points after saturation is beneficial to obtain background heat information
Injection volume	The injection volume to be injected into the ITC cell in a single injection	1st Inj: 1–2 µL rest of inj: 7–12 µL	The first injection is set to a lesser value to account for the backlash effect
Injection spacing	Time between two injections	250–500 s	The time between injections should be large enough to allow the DT signal to return to the baseline. The spacing also depends on the size of the peak
Titrate/titrant concentration	The concentration of the titrate/titrant that is input by the user which is also used for data analysis	Cell: 1.8 µM syringe: 42 µM	This example concentration was used for a 1:1 RNA–RNA interaction where the $\Delta H \sim 40$ kCal/mol <sup>d</sup>
ITC equilibration options	Equilibration options determine the modes of equilibration steps the VP-ITC needs to perform before an injection. The available mode are <i>No Check Temp</i> , <i>Fast Equilibrium</i> and <i>Automatic</i>	Automatic	(a) <i>No check temp</i> : the experiment starts at the current cell temperature as the entered value for the cell temperature is ignored (b) <i>Fast equilibration</i> : this setting will avoid the pre-stirring equilibration (c) <i>Automatic</i> : this will perform all equilibrations
Reference power	Amount of power that is supplied to the heater in the reference cell.	25–30 µCal/s	Generally setting a much higher value than is required may affect the sensitivity of the instrument
Initial delay	The delay between the start of data collection (baseline) and the first injection	60–100 s	A stable baseline needs to be developed prior to injections
Stirring speed	An optimum stirring speed is a value between efficient mixing and providing a stable a baseline	270–310 rpm	Higher stirring rates may cause high frequency noise in ITC data, whereas lower stirring rates may cause broader peaks
Feed back mode	The relative response times are indicated, <i>Fast</i> for faster response times, <i>None</i> mode will have the slowest response time but more sensitive for heat changes	Fast	For slow processes <i>None</i> mode is more suitable
Filter period	The data sampled from the cell feedback circuit is averaged over this period to produce a single data point	2 s	For slow reactions the filter period can be increased for better resolution of data

<sup>a</sup> Based on use with a VP-ITC from MicroCal. Specific parameter settings might need to be adjusted for use with other instruments.

<sup>b</sup> At higher temperatures the user must consider structural transitions of the titrate and titrant. Effects on the energetics of a reaction can be observed as much as 20 °C below the unfolding transition and these effects become increasingly significant as one gets closer to the  $T_M$  of any of the reactants or products.

<sup>c</sup> A terminal ratio of about three is obtained for a 1:1 interaction (i.e. The final excess ratio of the titrant over the titrate).

<sup>d</sup> The reaction was performed in 10 mM Hepes, 10 mM MgCl<sub>2</sub>, at 25 °C.

sitivity and setting it too low leads to artifacts from truncated peaks. In practice this parameter can be set to 25–30 µCal/s, a value that has been suitable for all of the RNA transitions we have studied. If one had particularly large injection volumes or an exceptionally exothermic reaction, one might alter this value, but one could just as easily use smaller injections or more dilute solutions instead.

#### 4.7. Data analysis

Having followed the advice above, at this point you hopefully have a suitable power curve for the titration of your system. Now it is time to convert that power curve into a thermogram.  $\Delta H_{obs}$  represents an aggregate measurement of all heat sources in the cell including some that are of interest and some that are not. The user needs to recognize and remove the unwanted background contributions during data analysis. Two types of background corrections are common. The first is a separate background run in which using the exact same ITC parameters one injects titrate into the ITC containing buffer only. The second method of performing a background correction involves including several injections at the end of the run in which no reaction occurs. There are pros and cons of each method. The independent run takes more time and material but is not subject to issues of non-specific binding effects. The use of terminal injections at the end of the titration ensures that the materials are exactly the same as those that were used to collect the primary data and that there are no inadvertent minor changes in the preparation of the buffers (such as from pipetting errors). Our primary rationale for using the terminal injections is to be certain that users collect the background data and that the background data cannot be separated from the ITC thermograms themselves.

In general, for a well-behaved system, the background heats are quite small. On occasion, however, the background injections can identify significant experimental artifacts. Salt and buffer discrepancies show up in these traces as do monomer–dimer equilibria (or higher aggregates) since upon dilution the aggregates may dissociate. If one suspects such behavior from one of the binding partners it can be confirmed by performing the ITC experiment at different concentrations or by switching the titrate and titrant to lower the concentration of the offending species.

Once raw data is corrected for background heats by either method above, the data are fit to a model that is appropriate to the system of interest. Most commercial ITC manufactures provide data analysis software containing the most common binding models. The software normalizes the heat of binding as a function of ligand concentration, automatically sets the baseline, performs a volume correction based on the dilution of the titrate during each injection and integrates each peak from the baseline before the binding model is selected by the user. Although most of the time the automated functions set the baseline appropriately, there may be instances where the user may need to do this manually if the data collection was non-optimal (e.g. due to poor baselines between injections resulting from slow binding kinetics). The integrated data then is used to determine  $n$ ,  $K_B$ ,  $\Delta H$ , by least squares minimization of Eq. (3).

The other aspect of data analysis has to do with fitting parameters. For a run-of-the-mill experiment, one will typically fit all three general parameters,  $n$ ,  $K_B$  and  $\Delta H$ . This provides a good level of quality control feedback on the experiment. If one is looking at an interaction that should be 1:1 but the stoichiometry is coming out to 0.7–0.8 (or 1.2–1.3), there is a reasonable likelihood that there is an experimental problem involving inaccurate concentration determination of one of the samples or an issue with alterna-

tive conformations that make a sub-population of the material unreactive.

In certain circumstances, one can fix parameters that are not accurately determined by the experiment. For instance, when the  $c$ -value is very high due to a very tight  $K_d$  (a situation that would be encountered if looking at nucleic acid secondary structure formation for instance), one can use ITC to obtain the reaction enthalpy but not the equilibrium constant. The software will allow you to lock parameters during fitting. Fixing the  $K_d$  at an independently determined value (such as from a thermal melting study) can get around the problem of an indeterminate value due to high  $c$ -values. Before doing this, however, one should be aware that there is an inherent danger as the analysis will only be as good as the independent measurement and only as reliable as the equivalence between the experimental conditions of the two studies.

#### 4.8. Special cases. What do I do if my $K_d$ is too tight?

In practice, the range of accessible interactions is not usually a problem for studies with most RNAs. Because RNAs are highly charged, the affinity for small molecules and proteins often can be shifted by altering the ionic conditions. For the rare case that might push the envelope, however, there are two approaches to extend the range of affinities accessible to ITC analysis. The first involves a single injection method where the titrant in the syringe is added to the cell in one long injection [37]. This process provides better definition of the transition for tight interactions and allows  $K_d$ s as low as 10 pM to be measured. The second approach involves a displacement titration methodology where a weak binding ligand is pre-bound to the macromolecule and the titration follows ligand exchange [2]. This technique requires that the user know the binding constants of the weakly binding ligand in advance from a separate experiment. The range of affinities accessible using this method is entirely dependent on the competitive binding ligands available for use.

## 5. Concluding remarks

The advantage that ITC holds on obtaining thermodynamics for binding interactions is that heat is the primary observable parameter that is a universal property of binding. Most other methodologies require modifications or labeling of the sample. One other advantage an ITC user enjoys over alternate binding methods is the ability to obtain the entire thermodynamic profile of a reaction from a single experiment without resorting to a concentration series (UV-melting) or a temperature series for a van't Hoff treatment. As modern ITC instruments continue to become more sensitive and versatile, sample requirements—currently the biggest disadvantage of ITC studies—will also decrease further. The ability to detect

the once neglected heat capacity changes in RNA conformational changes and to probe the thermodynamic contribution arising from complex molecular systems makes ITC an important tool for RNA biochemistry and biophysics.

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